Reprinted from PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE,
1965, v119, 492-495

2273

Acid and Salt-Soluble Collagen in Bovine Muscle.* (30218)

P. E. McCLAIN,† A. M. MULLINS, S. L. HANSARD, J. D. FOX AND R. F. BOULWARE
Louisiana Agricultural Experiment Station, Baton Rouge

Conventional methods for determination of collagen in muscle have been based on the assumption that intracellular constituents of muscle are solubilized by alkali solutions, while connective tissue collagen is insoluble. However, both dilute acid and dilute alkali soluble components of collagen have been reported(1,2), and a cold salt soluble fraction of collagen has been found which can be changed into mature collagen by warming to body temperature(3). These soluble collagens have been postulated to be metabolic precursors of insoluble mature collagens(4). Supporting reports in the literature show that soluble collagen can be altered by many factors including temperature, growth rate, nutrition, and by various endocrine preparations(5,6,7). Most studies on the soluble collagens have been with purified sources such as tendon, and cartilage. Therefore, knowledge of previously uninvestigated soluble components of collagens in bovine muscle would be beneficial for elucidating some of the chemical and physiological changes which occur in the conversion of living muscle tissue to edible meat, and for clarifying specific relationships of collagen to meat tenderness.

* This paper is published with approval of the Director, Louisiana State Univ. Agri. Exp. Station Baton Rouge. This work was supported by a contract with U. S. Dept. of Agriculture and authorized by Research and Marketing Act of 1946. The contract is being supervised by the Eastern Utilization Research and Development Division of Agricultural Research Service.

† Present address: Dept. of Food Science, Michigan State University, East Lansing.

Materials and methods. The fore shank muscles from 10 yearling steers were used in this study, primarily because of their high collagen content. All samples were removed within 15 minutes of dispatching and ground through 6.2 and 3.1 mm plates of an electric grinder. At 7 days post mortem, samples were taken from the *longissimus dorsi, semimembranosus* and *triceps brachii* muscles for Warner-Bratzler shear value measurements for use in correlation of tenderness with the soluble collagen fraction. A number of difficulties were encountered when existing methods were applied in collagen determination from bovine muscle tissue. The colorimetric method of Neuman and Logan(8) for hydroxyproline determination proved unreliable for muscle extracts. Two amino acids, tyrosine and tryptophan, have been reported to interfere with the colorimetric procedure(8). Tryptophan produced 0.7% as much color as hydroxyproline, but humin formation on acid hydrolysis of proteins eliminated this interference. Tyrosine, however, was not destroyed by acid hydrolysis and produced 1.5% as much color as did hydroxyproline. The recovery of an internal standard of hydroxyproline added to beef muscle extracts was never greater than 60% using this method. Tyrosine content of the salt-soluble extract was determined spectrophotometrically (Eastoe and Courts(9)), and found to contain 375 μg of tyrosine per ml. The observed interference of tyrosine on hydroxyproline in bovine muscle is shown in Table I. Although tyrosine contributed to color development in this procedure, it actually resulted in a sup-

TABLE I. Effect of Tyrosine on Absorbance* in Determination of Hydroxyproline in Bovine Muscle.

	Hydroxyproline, μg			
	0	5	10	15
Tyrosine, μg				
0	0	.112	.190	.325
400	.185	.245	.335	.440
600	.238	.310	.338	.440

* Values are absorbance readings taken at 557 mμ.

pression of color yield for hydroxyproline. It was determined that salt extracts contained approximately 2 μg per ml of hydroxyproline. At the 2 μg hydroxyproline—400 μg tyrosine level—a 36% reduction in total absorbance was evidenced. This would account for the failure to attain more than a 60% recovery of internal standards. The procedure of Martin and Axelrod(10) also proved to be unsatisfactory for muscle tissue.

The extraction procedure used in this study was a modification of the method of Gross(11) and Harkness(12). A 10 g sample was weighed into a 60 ml polyethylene centrifuge tube; 40 ml of 0.45 M sodium chloride was added, and samples were extracted 18 hours on a mechanical shaker. A 4 to 1 ratio of extractant to muscle tissue resulted in the greatest recovery of both acid- and salt-extractable collagen after 18 hours. All operations were carried out in a 3°C cold room. Samples were then centrifuged at 3,330 *g* for 60 minutes and passed through a coarse-sintered glass filter. Samples were washed with distilled water in the centrifuge (30 minutes at 3,330 *g*) and the rinse water was again filtered. Forty ml of citrate buffer, pH 3.8, was then added to the residue remaining after extraction. Citrate extraction was carried out for 18 hours, and the samples centrifuged and filtered as before.

Forty ml of the salt-soluble or acid-soluble filtrate were hydrolyzed with 40 ml of HCl for 15 hours at 120°C. Acid hydrolysis of muscle extracts was complete after 6 hours, and no reduction in yield was evident after 18 hours in the autoclave. Activated charcoal was added to the salt-soluble hydrolysates immediately after removal from the autoclave in order to remove humin. Samples were filtered and the charcoal washed several times with 6 N HCl. The hydrolysate from the acid-soluble fraction was then neutralized and assayed for hydroxyproline. However, for the salt-soluble hydrolysate, the salt concentration after direct neutralization, was too high for accurate hydroxyproline determination. It was, therefore, necessary to dry them on a rotary evaporator to neutrality. This procedure did not result in any detectable loss of hydroxyproline. Hydrolyzed samples were then diluted to contain 1 to 5 μg of hydroxyproline per 2 ml aliquot for hydroxyproline determination.

The acid- and salt-soluble fractions were assayed for hydroxyproline using a modification of Method II as described by Woessner (13). This procedure was developed to increase the sensitivity and stability of hydroxyproline chromogen and to eliminate interference from other amino acids in a variety of protein and tissue fractions. Preliminary determinations resulted in the recovery of internal standards of approximately 85%. According to Woessner, the addition of 5 mg of hydrolyzed protein to 5 μg of hydroxyproline resulted in a 5% decrease in absorbance. In an effort to lower the amino acid-hydroxyproline ratio of the salt soluble extracts, a preliminary extraction of muscle tissue with distilled water was attempted, and a recovery for added internal standard of 90 to 100% was accomplished. Therefore, this preliminary extraction was incorporated into the procedure for bovine muscle. More consistent results were obtained, however, using 45 ml of 70% perchloric acid diluted to 100 ml with water, instead of 27 ml recommended by Woessner. Preparations of p-dimethylaminobenzaldehyde from Matheson, Coleman and Bell were found to give the most satisfactory and consistent results. Certain lots of methyl cellosolve (ethylene glycol monomethyl ether) were unsatisfactory and it became necessary to pre-test each batch before being incorporated into the hydroxyproline procedure.

Results. Extractability of salt- and acid-soluble collagens at 30 minutes and at 7 days storage in steer and calf muscle tissue are shown in Table II. These data indicate salt-soluble collagen was reduced with sample

TABLE II. Salt- and Acid-Soluble Collagens* in Steer and Calf Fore Shank Muscles After 30 Minutes and 7 Days Storage.

	Steer		Calf	
	30 min	7 days	30 min	7 days
Salt-soluble collagen	23.2	8.8	19.0	8.1
Acid-soluble "	9.6	13.6	25.0	45.0
Total soluble "	32.8	22.4	44.0	53.1

* Expressed as μg hydroxyproline per g sample (fresh weight).

TABLE III. Mean Values for Salt- and Acid-Soluble Collagen in Bovine Shank Muscles.*

Tenderness groups	Shear value†	Salt soluble collagen‡	Acid soluble collagen‡
Less tender	35.59	115.08	76.62
Tender	21.11	96.34	43.40

* Samples extracted immediately after death.
† Mean shear values of *longissimus dorsi* muscles.
‡ μg hydroxyproline per g protein on a fresh-weight basis.

storage, which was in agreement with Gross (11). However, the quantity of acid-soluble collagen increased during this same storage period. Total soluble collagen was also observed to increase during storage in the calf samples.

Age differences were evident in quantities of salt- and acid-soluble collagens present. The quantity of salt-extractable collagen has been previously reported to increase with increasing animal age, with large quantities of soluble collagen occurring only in younger animals(11). Our data substantiated these reports. Steer muscle contained more salt-soluble collagen than did calf tissue; however, the total soluble collagen in calf muscle was greater.

Mean quantities of acid- and salt-soluble collagen in the fore shank muscle of carcasses used in this study are shown in Table III. No significant differences were observed between the 2 muscle tenderness groups in the amount of acid- or salt-soluble collagen. Since tenderness was not determined on the fore shank muscles, no conclusion concerning specific effect of soluble collagens on muscle tenderness was made. However, this study has revealed the presence of these soluble collagen fractions in muscle tissue. It has been widely accepted that collagen is not solubilized in 0.1 N sodium hydroxide. In fact, it is this lack of solubility which forms the basis for conventional methods of collagen analyses. Therefore, since these soluble components are not measured by conventional procedures, underestimation of the true collagen content of muscle may occur.

Discussion. Data have provided evidence that there may be differences in type and character of connective tissue not revealed by conventional methods for connective tissue determinations. Practically all previous research on connective tissue components influencing meat tenderness has been focused on the alkali-insoluble collagen and elastin moieties. Reports by Miller and Kastelic(14) have indicated that only 33% of the stroma was accounted for by fibrous elements. The remaining connective tissue was composed of previously uninvestigated connective tissue components such as ground substance, cellular inclusions, and soluble collagens. Perhaps the traditional approaches for studying the role of connective tissue on meat tenderness have been limited by a restricted concept of the morphological complexity of connective tissue. There remains the further compliation that chemical definitions for various components of connective tissue are still a matter for speculation. Gross analyses apparently do not reveal the presence of delicate chemical substances, nor elucidate their morphological character. Therefore, certain connective tissue constituents, though minimal in quantity, may well play a determining role in architectural aspects, and subsequently influence the tenderness of meat.

Summary. Salt- and acid-soluble collagens in bovine muscle tissue were investigated, utilizing modified procedures employed for purified collagen sources. The quantity of salt-soluble collagen in shank muscles decreased during post mortem aging, while acid-soluble collagen increased. Age differences were evident for acid- and salt-soluble collagen. The quantity of acid- and salt-soluble collagen in the fore shank muscles of cattle did not differ significantly between tender and less tender groups.

1. Lloyd, D. J., J. Phys. Chem., 1938, v42, 1.

2. Nageotte, J., Guyon, L., Am. J. Path., 1930, v6, 631.

3. Gross, J., Connective Tissue Symposium, 1957, Blackwell Publications, Oxford.

4. Orekhovitch, V. N., Shpikiter, V. O., ibid.

5. Gross, J., Highberger, J. H., Schmitt, F. O., Proc. Nat. Acad. Sci., Wash., 1954, v41, 1.

6. Houck, J. C., Jacob, R. A., Proc. Soc. Exp. Biol. and Med., 1960, v113, 692.

7. McIntosh, E. N., Acker, D. C., Kline, E. A., J. Agr. and Food Chem., 1961, v9, 121.

8. Neuman, R. E., Logan, M. A., J. Biol. Chem., 1950, v184, 299.

9. Eastoe, J. E., Courts, A., Practical Analytical Methods for Connective Tissue Proteins, 1964, Charles C Thomas, Springfield, Ill.

10. Martin, C. J., Axelrod, A. E., Proc. Soc. Exp. Biol. and Med., 1953, v83, 461.

11. Gross, J., J. Exp. Med., 1957, v107, 247.

12. Harkness, R. D., Marks, A. M., Muir, H. M., Neuberger, A., Nature and Structure of Collagen, Academic Press Inc., London, 1953.

13. Woessner, J. F., Jr., Arch. Biochem. Biophys., 1961, v93, 440.

14. Miller, M., Kastelic, J., J. Agr. and Food Chem., 1956, v4, 537.

Received February 23, 1965. P.S.E.B.M., 1965, v119.